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14. ABSTRACT
Stable fetomaternal microchimerism (MC), an exchange of fetal and maternal cells during pregnancy is a novel observation. Pregnancy-associated progenitor cells (PAPC) from male fetuses carry Y-chromosome markers. Fetal progenitors can contribute to normal regenerative processes, although their negative roles in autoimmunity and cancer were also reported. Since regeneration cycles facilitate progenitor integration, we postulated that post-pregnancy tissue remodeling in the mammary gland can integrate fetal progenitor-lineage. We proposed that disrupted progenitor differentiation may progress to breast cancer and finding Y-chromosome markers (SRY and DYS14) in clinical biopsies may confirm the hypothesis. In the first year we established the methodology for Y-marker detection including nested priming approaches, optimized DNA extractions from commercial breast cancer tissues and we also performed informative experiments to detect fetal elements in the samples. We presented our preliminary data in the AACR and ASCO Meetings in 2009. Progress has been hindered, however, by unforeseen non-specific signals that confounded our data. The extra measures (dUTP incorporation and UDG emzymatic pre-cleaning), an upgrade of the PCR-environment and the loss of a PCR cycler and a freezer slowed our progress. Since we need to re-investigate some of the samples to gain statistically relevant data, we asked for the extension of the project without additional cost or change in Specific Aims.

15. SUBJECT TERMS
microchimerism, mammary gland, fetal, progenitor, cancer, Y-chromosome, microdissection, PCR

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Annual Report

INTRODUCTION

The exchange of fetal and maternal cells during pregnancy results in the integration of fetal, multipotent progenitors in the mother. The established fetomaternal microchimerism (MC) is stable for life in most mothers (1) and these cells, called Pregnancy Associated Progenitor Cells (PAPC) represent a third type of progenitor pool (in addition to embryonic- and adult stem cells). The biological implications are significant. By homing in normal regenerative processes, they can rejuvenate stem cell pools and may contribute to the demonstrated lifespan advantage for women (1). On the other hand, their negative roles in autoimmunity and cancer have also been reported (2, 3). Y chromosome markers (from pregnancy with a male fetus) are indicators of the fetal lineage and detected in several maternal tissues (1). No data are available, however, on fetal progenitors in the human mammary gland, although the hormonal regeneration-degeneration cycles render the mammary gland a prime target for fetal progenitor integration.

We reasoned, therefore, that fetal progenitors are also present in the mammary gland and play a critical role in breast cancer. The biological outcome, however, is difficult to predict: (i) PAPC integration may correctly rejuvenate the stem cell pool and protects from breast cancer. This correlation has been shown with circulating MC cells (4). (ii) Alternatively, PAPC integration is initially beneficial, but immunological tolerance breaks down, as shown for autoimmune diseases (2). The developing persistent inflammation damages fetal progenitors that progress to breast cancer. Coincidentally, breast cancers also show high correlations with other autoimmune symptoms and develop extensive (auto?)-immune-inflammatory histology (5). Involvement of fetal cells in cervical cancer has been shown (3) and suggests a similar mechanism. To establish correlations with breast cancer and microchimerism, we proposed to detect Y-chromosome markers in breast cancer samples.

METHODS AND RESULTS

The methods we used and the results will be discussed according to the STATEMENT OF WORK portion of the application (*in parentheses and italics*).

("TASK 1 will establish the methodology for Y-marker detection in breast cancer samples from commercially available tissue-arrays. Methods for microdissection, DNA extraction and PCR design for Y-specific sequences will be optimized.
Months 1-3)
1. To optimize and implement the microdissection and DNA extraction methodology
Purchase positive control tissue arrays, for Y chromosome detection
CYBRDI prostate cancer tissue array"

The CYBRDI Company offers a wide selection of tissue microarrays in formalin-fixed paraffin-embedded (FFPE) format. To establish Y-marker detection in breast cancer samples, first we had to develop and optimize the methodology to detect Y-markers from a positive (male) tissue. We selected the following prostate cancer microarray from CYBRDI: CC19-01-003 that carries 63 prostate adenocarcinoma tissue cores from 33 patients with Gleason scores III-V. To extract the DNA from the tissue samples the slides were first deparaffinized and rehydrated using descending alcohol concentrations: Xylene for 2x10 min, then 100%, 95%, 70% and 50% ethanol treatments each for 10 min. The slides were dried under nitrogen to prevent oxydation.

"Microdissection by laser caption or mechanical instruments"

The tissue samples were dissected under a stereo-dissector microscope using three alternative ways: (i) manually, with a long loading tip on a micropipettor, in 2 ul Pickup Buffer (50 mM Tris pH 8; 100 mM EDTA); (ii) manually, with a hypodermic needle (Monoject, 27GA 0.4 mm x 12.7mm) in pickup buffer and (iii) laser-caption microdissector (Tufts Core Facility). The laser-caption microdissector did not worked efficiently on the CYBRDI slides, the adhesive mostly prevented harvesting. The samples were collected in 50 ul Digestion Buffer (1% SDS; 50 mM Tris pH 8; 100 mM EDTA) which was freshly supplemented with 200 ug/ml Proteinase K (0.5 ul 20 mg/ml stock added to 50 ul Digestion Buffer). The samples were digested at 50 ^{o}C for 2 days in a rotating oven.

"DNA extraction (Qiagen kits)"

First we tried to extract the DNA by using the DNeasy kit from the QIAGEN company. We had little luck and low yields and we realized that the amounts of the DNA preps were extremely low and the fragmented DNA did not bind efficiently to the mini-columns. We had good yields, however, by using standard phenol-chloroform extractions and ethanol precipitations. Comparative PCR analyses using dilutions of known genomic DNA preps indicated that the DNA collected from the samples were in the range of 0.2 to 2 ng per tissue core.

"2. PCR detection of Y-chromosome specific sequences
DYS14 and SRY specific primers
(nested amplifications, 2 areas per gene, total ~12 primers)"

Amplifying fragmented, damaged DNA from FFPE samples requires the design of short amplicons (<100bp). For primer design we used the NCBI Primer-BLAST Primer Design tool.

SRY gene primer design

One of the Y-chromosome specific genes is the SRY gene (NM_003140). The gene has similar sequences with the SOX3 gene on the X-chromosome (NG_009387), but or BLAST analysis showed that the sequence between 659-897 in the SRY coding area is Y-specific. We selected the following primers for nested amplification of SRY sequences:

PCR 1: (product: 104 bp)

YS679	5′GCA CCA GCT AGG CCA CTT AC	20N	Tm 60^{o}C
YS782r	5′CCA ATG TTA CCC GAT TGT CC	20N	Tm 60^{o}C

Nested PCR: (product: 71 bp)

YS685	5′CTA GGC CAC TTA CCG CCC AT	20N	Tm 63^{o}C
YS756r	5′CGC TAC AGC CAC TGG ACA AA	20N	Tm 63^{o}C

DYS14 marker primer design

The DYS14 sequence is a frequently used marker of the Y-chromosome and it is part of the TSPY1 gene (X06325). The published amplicons, however, require intact template sequences at lengths that extend the probable integrity of the FFPE DNA: 300 bp (6) and 200 bp (7). We selected the following primers using the NCBI Primer-BLAST Primer Design tool:

PCR 1: (product: 103 bp)

YD1082	5′CGA TCT GCG ACT GGG TCT	18 N	Tm 60^{o}C
YD1184r	5′CTG AGG CTG ACT GCA CTG AC	20 N	Tm 60^{o}C

Nested PCR: (product: 71 bp)

YD1082	*5′CGA TCT GCG ACT GGG TCT*	*18 N*	*Tm 60^{o}C see above!)*
YD1151r	5′ATG TGC GGA GAG TTG CTG GT	20 N	Tm 63^{o}C

To estimate the amount and quality of the extracted DNA, we needed a third, control primer pair on somatic chromosomes. For DNA quantitative and qualitative analyses (DNA test) we selected the APRIN gene on chromosome 13 since we had established primer pairs and PCR experience with the system in this laboratory:

PCR 1: (product: 95 bp)

Mx400a 5′GTA GGA AAC AAT TCA GTT AAC GGA AAG AAA ATG 33N Tm 67°C

Mx461b 5′GCC CTG CCA CAC ACA CGC 18N Tm 67°C

"This TASK will establish the optimal procedure to extract and analyze genomic DNA from tissue array samples and to detect Y-chromosome markers"

To establish the optimal procedure to detect Y-chromosome markers we first performed trial PCR reactions on DNA samples extracted from a male (prostate cancer) cell line, LNCaP. We used 1/1000 to 1/10,000 dilutions of the 1 ug/ul stock DNA preps. First we set up **SRY gene** detection and used Mg gradient at concentrations of 1.2, 1.4, 1.7 and 1.9 mM for optimization. Primer concentration was at 200 nM (YS679/YS782R) and we used 3 % DMSO to increase specificity. Amplifications were performed in 50 uls by 1 U Taq polymerase according to Invitrogen protocols through the following PCR program: denaturation: 94 °C 1:30 min; 5 touchdown cycles: 94 °C 25s, 54 °C 30s, 72 °C 25s; 33 cycles of 94 °C 25s, 52 °C 30s, 72 °C 25s, and extension at 72 °C 3 min and we also used untemplated control (H_2O). A representative ethidium-bromide band-pattern shown in Fig 1 established the optimal Mg conc. at 1.9 mM for this gene (arrow, single 104 bp band in lane 5).

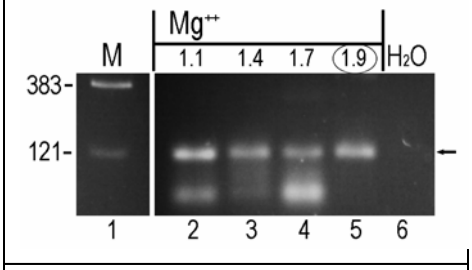

FIGURE 1. Mg-gradient PCR1 of the SRY gene on LNCaP template. Markers are in bp at the left. Mg concentrations and untemplated control (H2O) are indicated on the top. Specific product is labeled by arrow and optimal Mg conc. by a circle.

We followed similar methods to detect the **DYS14** marker by using the YD1082/YD1184R primers. A representative agarose-gel image in Fig 2 indicates that the optimal Mg conc. was established at 1.4 mM for the DYS14 marker (arrow, single 103 bp band in lane 3). We found, however, very intense secondary, non-specific bands.

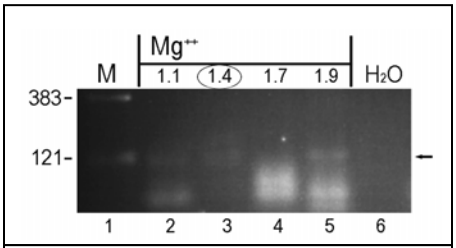

FIGURE 2. Mg-gradient PCR1 of the DYS14 on LNCaP template. Markers, controls and labeling are as in Fig 1.

To minimize non-specificity, next we performed **nested DYS14** amplifications. The PCR methods were similar as described above, but we used the YD1151R primer with YD1082 on 1/500 diluted lane-3 product of the first PCR, at 57°C touchdown and 25 cycles at 55 °C annealing temp. The expected 71 bp band was the major product at all Mg concentrations, as shown in Fig 3 (arrow) and we selected to work at 1.4 mM Mg in further studies, as the best option with minimal background (lane 4).

As an additional effort to eliminate non-specific bands, we replaced DMSO with **EnhancerX** (Invitrogen), a specificity enhancer compound. To make sure that the amplicons represent specific bands, we also introduced a

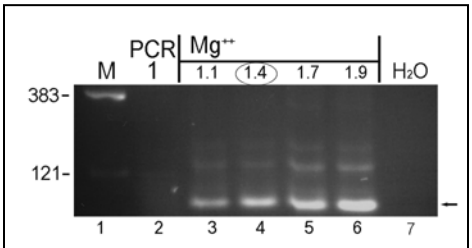

FIGURE 3. Mg-gradient nested-PCR on DYS14-PCR1 template. Markers, controls and labeling are as in Fig 1.

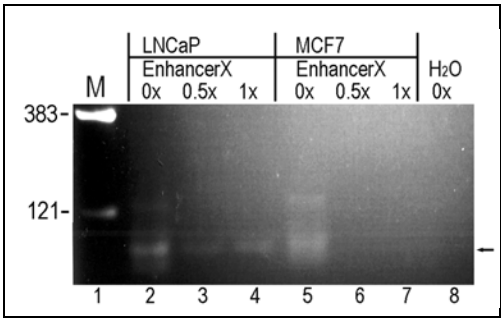

FIGURE 4. EnhancerX-gradient nested-PCR on DYS14-PCR1 template. Markers, controls and labeling are as in Fig 1. The non-template control was performed without EnhancerX for high sensitivity.

second negative control, DNA from MCF7, a female breast cancer line. We repeated **PCR1 for DYS14** with 0.5x, 1x and 2x EnhancerX concentrations and we found that 1x was optimal (not shown). For the EnhancerX-controlled **nested-DYS14 PCR** we set EnhancerX at 0.5x and 1x concentrations. A representative agarose-ethidium bromide image in Fig. 4 indicates that without EnhancerX multiple bands appeared (lanes 2 and 5), but EnhancerX repressed non-specificity and only the specific 70 bp band was present. Furthermore, the band was only detected in the male line (LNCaP), but was absent in the female line (MCF7).

To establish an optimal sensitivity/specificity ratio, next we wanted to identify an **optimal amplification level.** We were looking for the minimal necessary cycle number that generates the specific band, without overamplifying the background, under the established optimal Mg, EnhancerX and template conditions. We repeated the previous nested PCR reactions at 1.4 mM Mg and 1x EnhancerX levels on both the male (LNCaP) and the female (MCF7) templates with non-templated controls. We took, however, 8 ul aliquots of the reactions at cycles 20, 24, 28, 32 and 35 and run them separately in agarose electrophoreses. A representative ethidium-bromide image in Fig. 5 shows strong DYS14 signal in the male sample (LNCaP), but at 35 cycles faint non-specific bands also appeared in the MCF7 and non-templated controls. Since the MCF7 signal intensity at cycle 35 is comparable with the LNCaP signal at cycle 24, the difference (11 cycles) indicates a roughly 2000-fold lower detection of the Y signal in MCF7 and water, at the level of technical background or "noise".

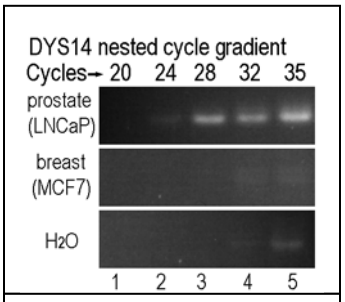

FIGURE 5. DYS14 nested cycle gradient PCR. Cycle numbers are on top. Templates are indicated at left.

PROBLEMS AND SETBACKS At this point of progress, however, our main PCR equipment failed and the samples boiled out possibly contaminating the lab environment. We switched to another PCR facility, but our efforts to optimize SRY gene detection has not been successful so far. The primary PCR repeatedly produced positive signals in every negative control we tried (not shown).

To prevent further contaminations, we established the highest level of PCR specificity by using dUTP incorporation and Uridyl DNA Glycosylase (UDG). In this system the PCR product incorporates deoxy-uridyl, which, in turn, is recognized by UDG. The enzyme destroys carry-over contaminating DNA from previous PCR products, but does not affect natural templates. In the rest of the experiments the dUTP-UDG system was used.

The UDG screen, however, did not help to establish specific SRY marker detection. We finally concluded to resynthesize the SRY core primer set and design a new set for PCR1. The new primers are under investigation and we plan to optimize a nested detection system also for the SRY marker. Another incident, by loosing a freezer affected our core reagents and several series of extracted DNA preps. Re-ordering the reagents and replacing the samples are under way. Altogether, these incidents caused considerable setbacks and significant loss of time.

Nevertheless, we progressed in isolating and analyzing DNA from both prostate and breast cancer tissue array samples and performed Y marker analyses.

(*TASK 2 will analyze a number of breast cancer samples and normal controls for the presence of fetal progenitor microchimerism to establish correlation patterns*
Months 4-10
1. Microdissection of breast cancer and normal samples and extraction of genomic DNA
Purchase of breast cancer tissue arrays CYBRDI, Millipore
Microdissection by laser caption or mechanical instruments
DNA extraction - Qiagen kits)

In the first part of this Task we optimized our DYS14 detection method in samples extracted from FFPE tissue arrays. This is a challenging approach since the extracted DNA is mostly fragmented, damaged and the preparations contain PCR inhibitors. Optimization required positive samples, so we used tissue arrays from male patients, prostate cancer arrays we ordered and processed earlier. We also ordered and processed breast cancer arrays from CYBRDI (CC08-03-001 with 63 cores from 38 cases). We extracted DNA samples from both prostate and breast specimens by microdissections described above and by using phenol-chloroform and ethanol precipitation, following standard techniques.

The amounts of purified DNAs were low and routine methods to test DNA (OD_{260} spectrophotometry or agarose ethidium bromide electrophoresis) were not sensitive enough. We used therefore a PCR approach to test DNA preps (**qualitative DNA tests**). The APRIN gene is studied in this laboratory and we used one of the available genomic primer pairs (see above) to assess DNA quality and quantity. Tissue array processing and DNA extractions were performed from both prostate and breast cancer tissue array slides. The final ethanol pellets were dissolved in 20 ul DNA Buffer (1 mM TRIS-HCl pH 8; 0.1 mM EDTA). For DNA detection we used 5 ul extracted DNA in PCR reactions described above using the Mx400a/Mx461b primer pairs for APRIN in 45 cycles. A representative analysis of 10 samples is shown in Fig 6 and indicates that DNA extraction was successful in about 50% of the samples (panel A samples 1, 4; and panel B samples 7, 8, 9).

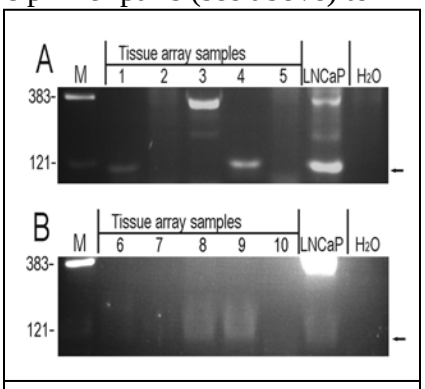

FIGURE 6. Qualitative and quantitative test (APRIN PCR) of DNA extracted from fixed tissue array cores. Sample numbers, as well as positive (LNCaP) and negative (H_2O) controls are indicated on the top. Markers are in base pairs at left.

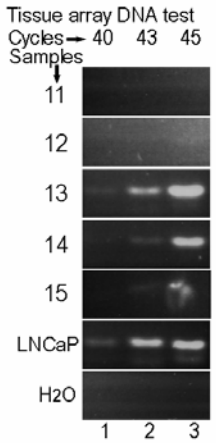

FIGURE 7. Quantitative cycle gradient PCR on tissue array core DNA. Cycle numbers are on top, sample numbers are indicated at left.

For **quantitative DNA tests**, we performed cycle gradient PCRs. The above PCR reactions on tissue array DNAs were interrupted at cycles 40, 43 and 45, and 8 ul aliquots were taken. The aliquots were run separately in a 2.5% gel and the results on Fig 7 indicate that 3 out of 5 preps contained amplifiable DNA (samples 13, 14 and 15). By comparing relative band intensities, absolute DNA contents could be assessed through the LNCaP results where the DNA load was known. These types of estimates suggested that the DNA yield from the tissue array slides varied between 0.2 ng to 2 ng per sample.

Now that we knew that DNA extractions from the fixed, embedded cancer micro-samples were successful, we tested if we could **detect the Y chromosome through the DYS14 marker.** First we used a series of **prostate cancer samples as positive controls** to establish the right methodology.

After DNA extractions from the samples, we performed both the DNA testing and DYS14 marker PCR reactions following the protocols described above. A representative image in Fig 8 shows good correlation between DNA detection and Y-chr. marker positivity. The results suggest that the extracted DNA is a good template for Y-chromosome marker analyses.

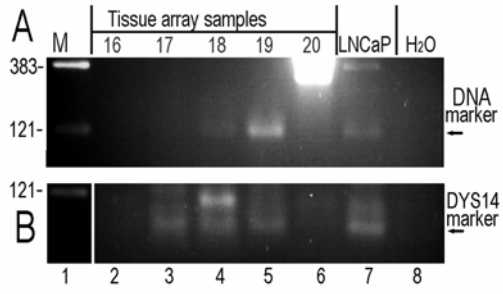

FIGURE 8. Y-chromosome assays (DYS14 marker) on DNA samples extracted from prostate cancer tissue arrays. Panel A, DNA assay; panel B, DYS14 assay. Arrows indicate PCR products.

By applying the established methodology, we also performed the **DYS14 marker assay on a series of breast cancer samples**. The low signal required 33 cycles in the nested PCR that also enhanced non-specific bands. A typical result is shown in Fig. 9. Sample #1 was a normal breast DNA extract, #2 to #10 were breast cancer samples. The single band in the male control (LNCaP) identified the specific signal, which is indicated by the arrow and labeled by an asterisk also in the breast cancer patterns. We found low level Y-representation in the normal breast. In 6 of the 9 cancer samples, however, the Y signal appeared to be enhanced.

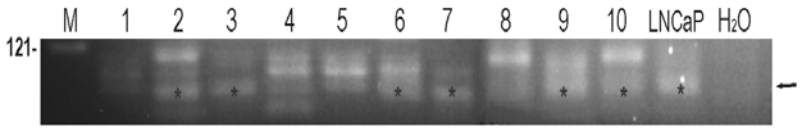

FIGURE 9. Y-chromosome assays (DYS14 marker) on DNA samples extracted from breast cancer tissue arrays. Arrow indicates the specific PCR product. Sample #1 is normal tissue; #2-#10 are breast cancer samples. LNCaP, Y-chromosome positive control. Marker band is indicated at left in base pairs.

These results represent the first data on the presence of Y chromosomal sequences in breast cancer. The data, however, need further confirmation by (i) establishing a second Y-marker through the SRY gene assay; (ii) by further optimizing the DYS14 signal to eliminate non-specificity; (iii) once single band specificity is worked out, we will establish Real-Time Quantitative PCR methods for accurate quantitative data; and (iv) we will analyze a larger number of normal and cancer tissues to increase the statistical power of the data. To achieve these goals we need more time and asked for an extension of the project for one more year, without additional cost.

(2. Y-chromosome marker identification

Real Time PCR kits, SYBR-Green-technology, QIAGEN or Invitrogen

Real-Time PCR software analysis to generate quantitative values)

This part of the project awaits further optimization of the DYS14 signal to eliminate non-specificity. When single band specificity is worked out, we will establish Real-Time Quantitative PCR methods as mentioned above.

(TASK 3 will analyze the data, we will use statistical analyses to establish and verify correlations with breast cancer.

Months 10-12)

Data analysis has already been under way, but we need more Y-chromosomal data from breast cancer and normal mammary gland samples. This part of the project will be done after completion of the experimental phase in the no-cost extension period.

KEY RESEARCH ACCOMPLISHMENTS

1 Established the methodology to extract amplifiable DNA from fixed, paraffin-embedded tissue array cancer samples.

2 Worked out the technology for the DYS14 marker assay to detect the presence of Y-chromosome sequences in these DNA preparations.

3 The results represent the first data on the presence of Y chromosomal sequences in breast cancer.

4 The data suggest, for the first time, that fetal cells are able to integrate into the mammary gland.

5 This integration event appears to coincide with breast cancer.

6 The high representation also suggests potential involvement of fetal progenitor cells in the pathomechanisms of breast cancer.

REPORTABLE OUTCOMES

Published Abstract #1:

Geck, P., Denes, V., Pilichowska, M., Makarovskiy, A.N. and Carpinito, G.A. (2009) Translational disequilibrium as an interference marker to study miRNA and methylation silencing of APRIN, a stem cell regulator in breast cancer microchimerism (Published Abstract). In: **Journal of Clinical Oncology,** Vol 27, (June Supplement), 2009 (ASCO Annual Meeting Proceedings). (45th ASCO Annual Meeting, May 29-June 2, 2009; Orlando, FL)

Presentation of preliminary data in the 45th ASCO Annual Meeting, May 29-June 2, 2009; Orlando, FL

Published Abstract #2:

Denes, V., Pilichowska, M., Makarovskiy, A.N., Carpinito, G.A. and **Peter Geck**. (2009) A stem cell role for cohesins? APRIN(Pds5B) is involved in stem cell regulation, fetal microchimerism and silenced in many cancers (Published Abstract). In: **Proc. Amer. Assoc. Cancer Res.** Vol. 50, 09-AB-7763 (Annual Meeting of the American Association for Cancer Research, Apr 18-22, 2009; Denver, CO)

Presentation of preliminary data in the Annual Meeting of the American Association for Cancer Research, Apr 18-22, 2009; Denver, CO

CONCLUSIONS

Our data strongly suggest the presence of Y chromosomal sequences in breast cancer. The implications are several fold. At a technical level, we showed that it is technically possible to gain DNA from fixed tissue array samples. Moreover, the DNA is in sufficient quantity and quality to perform multiple genetic analyses. The most important biological implication is, however, that fetal cells appear to be able to integrate into the mammary gland. Our preliminary data also suggest that this integration event appears to coincide with breast cancer. Finally, the high representation of the Y signal may also imply that fetal progenitor cells may be potentially involved in the pathomechanisms of breast cancer. The requested no-cost extension of the project will help to work out more details of the technology and to generate statistically solid data.

REFERENCES

1. Nguyen Huu S, Dubernard G, Aractingi S, Khosrotehrani K. (2006) Feto-maternal cell trafficking: a transfer of pregnancy associated progenitor cells. Stem Cell Rev. 2:111-6. PMID: 17237549

2. Lissauer D, Piper KP, Moss PA, Kilby MD. (2007) Persistence of fetal cells in the mother: friend or foe? BJOG. 114:1321-5. PMID: 17949373

3. Cha D, Khosrotehrani K, Kim Y, Stroh H, Bianchi DW, Johnson KL. (2003) Cervical cancer and microchimerism Obstet Gynecol.102:774-81. PMID: 14551008

4. Gadi VK, Nelson JL. (2007) Fetal microchimerism in women with breast cancer. Cancer Res. 67:9035-8. PMID: 17909006

5. Stewart TH, Heppner GH. (1997) Immunological enhancement of breast cancer. Parasitology 115:S141-53. PMID: 9571699

6. Yang, YH., Yoo, HS, and Kim, IK. (1995) Prenatal fetal sex determination from maternal peripheral blood using polymerase chain reaction. Yonsei Med J. 36:361-366.

7. Lambert NC, Lo YM, Erickson TD, Tylee TS, Guthrie KA, Furst DE, Nelson JL. (2002) Male microchimerism in healthy women and women with scleroderma: cells or circulating DNA? A quantitative answer. Blood. 100:2845-51

APPENDICES

Appendix 1. Published Abstract at the 45th ASCO Annual Meeting, May 29-June 2, 2009; Orlando, FL

Geck, P., Denes, V., Pilichowska, M., Makarovskiy, A.N. and Carpinito, G.A. (2009) Translational disequilibrium as an interference marker to study miRNA and methylation silencing of APRIN, a stem cell regulator in breast cancer microchimerism (Published Abstract). In: **Journal of Clinical Oncology,** Vol 27, (June Supplement), 2009 (ASCO Annual Meeting Proceedings). (45th ASCO Annual Meeting, May 29-June 2, 2009; Orlando, FL)

BACKGROUND Gene silencing is universally observed in cancer and involves epigenetic promoter DNA methylation. We found that a cohesin associated factor, APRIN (Pds5B), an embryonic stem cell regulator was frequently silenced in breast cancer clinical samples. Surprisingly, in 40% of these samples DNA methylation was not involved. Furthermore, in some breast cancer cell lines APRIN was silenced only at the protein level without transcript downregulation or promoter methylation. This "translational disequilibrium" has been frequently reported in cancer with other proteins, but without mechanistic explanations. Recent results with RNA interference indicate that gene repression through microRNAs (typically mismatched) does not include transcript degradation and mostly translational. We propose, therefore, that the puzzling translational disequilibrium phenomenon represents a new form of epigenetic silencing by miRNA mechanisms. We aim (i) to verify miRNA epigenetics of APRIN silencing in breast cancer cell lines; (ii) to study clinical breast cancer samples for APRIN translational disequilibrium and methylation vs. miRNAs mechanisms; and (iii) to investigate if miRNA silencing of APRIN specifically affects a fetal embryonic stem cell pool in breast cancer (microchimerism).

METHODS (i) We used miRNA mimics and miRNA inhibitors in breast cancer cell lines to verify specific miRNA involvement in APRIN silencing. (ii) We used immunohistochemistry with bisulfite converted DNA for methylation and microdissected RNA for microRNA interference studies from 56 clinical breast cancer samples. (iii) We used Y-chromosome markers on microdissected DNA for fetal microchimerism studies.

RESULTS (i) We found that in breast cancer cell lines with APRIN translational disequilibrium a set of microRNAs correlate with APRIN silencing. (ii) We found miRNA related mechanisms in about 35 percent of breast cancer samples where APRIN was silenced and (iii) APRIN may specifically affect stem cells of fetal origin in the mother's mammary gland and contribute to cancer.

CONCLUSIONS The novel miRNA-based mechanism maybe a new epigenetic factor of gene silencing in cancer. We experimentally confirmed a set of APRIN specific miRNAs and established preliminary correlations with fetal microchimerism in breast cancer.

Appendix 2. Published Abstract at the Annual Meeting of the American Association for Cancer Research, Apr 18-22, 2009; Denver, CO

Denes, V., Pilichowska, M., Makarovskiy, A.N., Carpinito, G.A. and **Peter Geck**. (2009) A stem cell role for cohesins? APRIN(Pds5B) is involved in stem cell regulation, fetal microchimerism and silenced in many cancers (Published Abstract). In: **Proc. Amer. Assoc. Cancer Res.** Vol. 50, 09-AB-7763 (Annual Meeting of the American Association for Cancer Research, Apr 18-22, 2009; Denver, CO)

PURPOSE Cohesins are not known to play a primary role in the pathomechanism of cancer. We found that APRIN (PDS5B-Precocious Differentiation of Sister-chromatids 5B, a cohesin-associated factor) is silenced in a portion of breast, prostate and ovarian cancers. Surprisingly, recent APRIN knockout studies also suggest an APRIN role in birth defects (Cornelia de Lange Syndrome). Since both cancer and birth defects have been shown to correlate with disrupted stem cell programs, the common mechanism appears to be stem cell related. We investigated the hypothesis, therefore, that APRIN may function in stem cell differentiation. Our objectives were (i) to study the APRIN mechanism in embryonic stem cell models; (ii) to investigate the mechanisms of APRIN silencing in cancer; and (iii) to establish if APRIN silencing in fetal (embryonic) stem cell microchimerism correlates with breast cancer.
EXPERIMENTAL PROCEDURES (i) We used transfection and RNA interference in the P19 murine embryonic stem cell model to study APRIN in differentiation. Microarray studies established the APRIN transcriptome in breast cancer cells. (ii) Immunohistochemistry detected APRIN silencing in clinical breast and prostate cancer samples. Microdissected DNA preparations were bisulfite converted for promoter methylation analyses and microdissected RNA preparations were used to study microRNA interference in silencing. (iii) We tested microdissected DNA for Y-chromosome markers to establish fetal microchimerism in breast cancer.
SUMMARY OF RESULTS (i) We show the molecular mechanism of APRIN and demonstrate that APRIN is critical in embryonic stem cell differentiation. APRIN knockdown established the APRIN-regulated transcriptome (stem cell signalling pathways, e.g. Wnt, E-Cadherin, Oct4, Mash1 etc.). (ii) We show methylation hot-spots in the APRIN promoter and 20-80% methylation in our breast and prostate cancer pool. In addition, we found that APRIN-specific microRNA upregulation correlated with APRIN silencing in breast cancers. (iii) APRIN controls embryonic stem cell differentiation, yet it was silenced in adult cancers, suggesting that APRIN may block stem cells of fetal origin in the mother's mammary gland (fetal microchimerism). We detected Y-chromosome markers (microchimerism) in a number of breast cancer tissues and preliminary data indicate correlation with APRIN silencing.
CONCLUSIONS (i) APRIN, a cohesin-associated factor appears to have a critical role in embryonic stem cell differentiation, a surprising new function for cohesins. (ii) We found a novel epigenetic mechanism: RNA interference (miRNAs) maybe an epigenetic factor of gene silencing in cancer. (iii) APRIN silencing appears to correlate with fetal microchimerism in breast cancer, suggesting that disrupted differentiation of the fetal resident population in the mammary gland contributes to cancer.